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(71)Applicant

Wako Pure Chemical Industries, Ltd.

1-2, Doshomachi 3-chome, Chuo-ku, Osaka-shi, Osaka, Japan

(72)Inventor

KOBAYASHI, Noribumi

c/o Wako Pure Chemical Industries, Ltd., Osaka Laboratories,

6-1, Takata-cho, Amagasaki-shi, Hyogo, Japan

(72)Inventor

SUZUOKI, Jun

c/o Wako Pure Chemical Industries, Ltd., Osaka Laboratories,

6-1, Takata-cho, Amagasaki-shi, Hyogo, Japan

[TITLE OF THE INVENTION] ANTIBODY AGAINST CHOLESTERYL ESTER TRANSFER PROTEIN

[ABSTRACT]

[Object]

It is an object of the present invention to provide an antibody against cholesteryl ester transfer protein, which has high titer to cholesteryl ester transfer protein (CETP), low crossing-over activity to measurement preventing materials or other proteins in blood, and recognizes a stable epitope in CETP molecules, and to provide a simplified and high accurate immunoassay method for CETP measurement of a sample by use of said antibody, and an assay reagent for CETP which can be used for the immunoassay method.

[Solution]

An immunoassay method of CETP in a sample as well as an assay reagent for said method, in which a monoclonal antibody with high titer to CETP devoid of any cross-reactivity to measurement preventing materials in blood and a sample pre-treated with SDS are reacted with each other in the presence of SDS to form the antigen-antibody complex.

[Claims]

[Claim 1] A monoclonal antibody that has high titer to cholesteryl ester transfer protein and lacks crossing-over activity to measurement preventing materials in blood.

[Claim 2] An antibody reacting specifically with cholesteryl ester transfer protein treated with dodecyl sodium sulfate.

[Claim 3] A monoclonal antibody reacting specifically with cholesteryl ester transfer protein treated with dodecyl sodium

sulfate.

[Calim 4] A method for immunologically measuring cholesteryl ester transfer protein, characterized in that reacting a sample previously treated with dodecyl sodium sulfate and the antibody according to 2 or 3 in the presence of dodecyl sodium sulfate to generate an antigen-antibody complex.

[Claim 5] A reagent for measuring cholesteryl ester transfer protein characterized in that said reagent comprises an antibody of any one of claims 1 to 3.

[Detailed Description of the Invention]

[Technical Field of the Invention]

The present invention relates to an antibody to cholesteryl ester transfer protein (hereinafter abbreviated to as CETP), an immunoassay method of CETP using said antibody, and a reagent used for the immunoassay method for measuring CETP.

[Description OF the Prior Art]

CETP is a protein which has an activity of pulling out a cholesteryl ester (CE) from high-density lipoprotein (HDL) and transfers it to very-low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL), and thus plays a primary role in so-called cholesteryl ester transfer pathway which is a metabolic pathway of cholesterol.

The CETP is the protein of which absolute purification technique is established in last half of 1980 era [Jarnagin, A.S., et. al., Proc. Natl. Acad. Sci USA 84, 1854-1857 (1987); Hesler, C. B., et. al., J. Biol. Chem. 262, 2275-2282 (1987);

Kato, H., et. Al., J. Biol. Chem. 264, 4082-4087 (1989)]. According to the analysis by use of m-RNA, it is known that the CETP is mainly biosynthesized in lever, spleen, adipose tissue, small intestine and adrenal and exists in blood within an organism. It is known that human CETP is a sialic acid containing glycoprotein with the molecular weight of approximately 74,000 and is composed of 467 amino acid residues of which molecular weight is calculated as 58,000 when the sugar chains are completely removed. Further, CETP is characterized by its significantly higher hydrophobic property than those of other proteins in blood because the constituent amino acids of CETP include hydrophobic amino acids by 44%.

Much of the in vivo action of CETP is not yet understood. However, there is a report indicating that the production of CETP by macrophages or smooth muscle cells in arterial cell wall is related to the elimination of cholesterol from arterial wall [Stein, O., et. Al., Arteriosclerosis 6, 70-78 (1986)]. In the case, it is thought that CETP acts as anti-arteriosclerotic.

On the other hand, the activity of CETP in blood varies depending on animal species. For example, the CETP activity is very strong in rabbit but the substantial activity is not observed in rat, sheep etc. The activity of CETP in human is moderate among them.

It is known that the animal species with strong CETP activity tends to be prone to arteriosclerosis upon cholesterol loading, and on the contrary, in the animal species with week CETP activity, arteriosclerosis is hard to be induced. Further, it

is known that in the case of HDL known to as a carrier of cholesterol in the pathway reversing transfer of cholesterol from tissue cell to lever, there is negative relationship between the blood level of cholesterol in HDL (HDL cholesterol) and ischemic heart disease. On the contrary, in the case of LDL which is a carrier of cholesterol directed to tissue cell, there is a positive relationship between the blood level of cholesterol in LDL (LDL cholesterol) and ischemic heart disease. In inherited CETP deficient patients, an increased blood-HDL cholesterol level due to lack of CETP activity causes high HDL cholesteremia, though it is recognized that much patient family line is in longevity [Inazu, A. et. al., N. Engl. J. Med. 323, 1234-1238 (1990)]. It is assumed that the patients are not prone to arteriosclerosis due to lack of CETP and accordingly life-style diseases such as arteriosclerosis are scarce among them. Further, it was reported that the HDL level decreased by 20 to 30% in transgenic mice transfected with human CETP genes in order to enhance the CETP activity in the animals [Agellon, L. B., et al., J. Biol. Chem. 266, 10796-10801(1991)], which caused atherosclerosis when the mice were given foods with high cholesterol, indicating that CETP may accelerate arteriosclerosis [Marotti, K. R. et al., Nature 365, 73-75(1993)]. Furthermore, in patients with protopathic biliary cirrhosis (PBC), increased CETP activity is also observed. These findings demonstrate the significance of CETP measurement as an index of PBC.

As mentioned above, it is considered that correct measurement of CETP activity or CETP concentration is extremely importance in the research area of arteriosclerosis

associated diseases and hepatic disease, or in the area of diagnosing these diseases, and then various measuring methods including those measuring CETP activity and CETP concentration have been suggested.

For measuring CETP activity, for example, a method for calculating CE activity is known wherein a sample is added to a reaction solution containing a donor lipoprotein (HDL) labeled with radioisotope at the cholesteryl ester (CE) moiety and acceptor lipoproteins (VLDL, IDL, and LDL) at their respective constant rates, and the mixture is incubated at a constant temperature for a constant time, and then the donor lipoprotein and the acceptor lipoproteins are separated by use of precipitation method such as ultra centrifugal method or heparin-manganese method and each radioactivity is measured [H. Arai & K. Inoue, The lipid 2/2, 183-195 (1991)]. However, the activity measuring method is troublesome in that it needs high technology and special equipments and it also needs too much time for measuring because the operation is very complicated.

Further, an immunoassay method by use of antibody for measuring CETP concentration was reported [Y. L. Marcel, et al., J. Clin. Invest. 85, 10-17 (1990), H. Mezdour, et al., Clin. Chem. 40/4, 593-597 (1994)]. However, there was a problem that most of antibodies used for such immunoassay method recognized the vicinity of extremely unstable lipid transfer catalytic site in the C-terminus of CETP and did not recognize other stable epitopes existing in the CETP molecules [Y. L. Marcel, et al., J. Clin. Invest. 85, 10-17 (1990)], meaning that the measurement of CETP concentration becomes

impossible as the lipid transfer catalytic activity of CETP in the sample is lost. Further, there was a problem that a purified CETP as well as CETP in a sample was extremely unstable. That is, the lipid transfer catalytic activity is easily lost by freeze-thaw or storage at 4°C, and therefore the purified CETP or samples must be stored under extremely low temperature conditions as below as -80°C for preventing them from being deactivated. The above has raised a difficulty in providing a purified CETP specimen. This has also raised a difficulty in measuring the activity or the concentration of CETP because the reliability of the measurement should be significantly affected by the above-mentioned property of CETP. Accordingly, there was a great difficulty to develop a commercially available CETP measuring kit.

Further, such conventional antibodies were found to show a considerable cross-reactivity to other proteins in blood such as albumin, immunoglobulin, and fibrinogen. Therefore, to accurately analyze CETP in blood was extremely difficult when the antibodies as above were used.

Further, to solve these problems, another proposed method is that an antibody is raised against a synthetic immunogen which is, for example, chemically synthesized from known amino acid sequence of CETP to obtain the antibody capable of recognizing an epitope which is stably in the CETP molecule and does not show any cross-reactivity to other proteins in blood or measurement preventing materials. However, the antibodies obtained according to this the method had much trouble in designing the CETP measuring system because its reactivity to CETP was low even though its cross-reactivity

to other proteins in blood or measurement preventing materials was low.

[Problems to be Solved by the Invention]

It is an object of the present invention to provide an anti-CETP antibody, which has high titer to CETP, its cross-reactivity to protein in blood or measurement preventing materials is low, and recognizes epitope existing in CETP molecule, a simplified and high-precision immunoassay method of CETP in sample by use of said antibody, and a CETP measuring reagent for the method.

[Means for Solving the Problems]

The present invention is (1) an invention of monoclonal antibody with high titer to CETP and without cross-reactivity to measurement preventing materials existing in blood.

Further, the present invention is (2) an invention of antibody reacting specifically with CETP treated with sodium dodecyl sulfate (SDS).

Furthermore, the present invention is (3) an invention of monoclonal antibody reacting specifically with CETP treated with SDS.

Further, the present invention is (4) an invention of immunoassay method for CETP in a sample, in which antigen-antibody complex is generated by reacting the sample pre-treated with SDS with the antibody of (2) or (3) in the presence of SDS.

Furthermore, the present invention is (5) an invention of CETP measuring reagent, including antibody of any one of the above-mentioned (1) to (3).

That is, as a result of keen research for obtaining the

anti-CETP antibody which has high titer but a low level of cross-reactivity to other proteins in blood or measurement preventing materials while recognizing an epitope existing stably in CETP molecule, the inventors found that it is possible to obtain an anti-CETP antibody which recognizes an epitope existing stably in CETP molecule, but shows no cross-reactivity to other proteins in blood or measurement preventing materials and thus has high titer to CETP, especially CETP treated with SDS, by using CETP as immunogen which was treated with SDS in advance, which makes the immunogen negatively charged. Further, the inventors reached to complete the invention by the finding that they could easily, specifically and precisely measure the presence of CETP in a sample if the sample is pre-treated with SDS and reacted with said antibody in the presence of SDS which strongly inhibits antigen-antibody reaction in usual cases.

The anti-CETP antibody of the present invention is obtained by using as the immunogen, a pre-treated CETP with SDS to make CETP in negatively charged. Thus obtained antibody has high titer to CETP especially CETP treated with SDS, and has no cross-reactivity to other proteins in blood such as albumin, immunoglobulin, and fibrinogen which are the examples of the measurement preventing materials. The preset antibody also includes including those with a low level of cross-reactivity, that is, an antibody without a problem of cross-reactivity from a practical standpoint in CETP measurement using said anti-CETP antibody. Further, the anti-CETP antibody reacting with the CETP treated with SDS of the present invention includes those antibodies insofar as

they can show some specificity to CETP treated with SDS. However, it is preferred that the antibody has no reactivity or only has a limited reactivity to untreated CETP and also shows no cross-reactivity or a low level of cross-reactivity to measurement preventing materials in blood such as albumin, immuno-globulin and fibrinogen. In other words, the antibody of the present invention can be used without the problems in CETP measurement using said anti-CETP antibody in actual use. The anti-CETP antibody is not particularly limited with regard to the origin insofar as the properties as above-mentioned are fulfilled, and a polyclonal antibody and a monoclonal antibody may be included.

Examples of the method for obtaining the polyclonal antibody with such properties include a method wherein, according to the conventional protocol, the anti-CETP polyclonal antisera is obtained from animals such as horse, cow, sheep, goat, rabbit, guinea-pig, rat, and mouse that are immunized with a purified and SDS pre-treated CETP, and the polyclonal antibody is purified from the antisera by using an affinity column in which CETP treated with SDS is immobilized.

Yet, examples of the method for obtaining the anti-CETP monoclonal antibody of the present invention includes: a hybridoma is produced by fusing a sensitized cell such as spleen cell or lymph cell from animals such as horse, cow, sheep, goat, rabbit, guinea-pig, rat, and mouse immunized by the immunogen with a permanent cell line such as myeloma cell by using well-known cell fusing technology developed by Keller, Milstein et al., wherein the immunogen as used has been prepared by treating CETP with SDS to make the protein negatively charged.

Then, the hybridoma which produces a monoclonal antibody reacting specifically with CETP treated with SDS is selected and the said hybridoma is cultured in a medium or in the abdominal dropsy of animals by injecting the hybridoma into the abdominal cavity. Accordingly, the objective monoclonal antibody is obtained from said culture or abdominal dropsy. Another example is such that a cell producing an antibody with above-mentioned properties is created by well known genetic recombination technology [Eur. J. Immunol., 6, 511 (1976)] and culturing the cells.

In the method for obtaining the anti-CETP antibody, the SDS treated CETP using as the immunogen is prepared by fractionating human blood plasma to collect a fraction of the specific gravity of above 1.21 by using, e.g. ultra centrifugal method such as concentration gradient ultra centrifugal method or continuous isopyknic ultra centrifugal method, and purifying the immunogen by using a well-known purifying method such as affinity chromatography, ion-exchange chromatography, gel filtration, ammonium sulfate fractionation, and salting-out. By such purifying methods, so-called crude CETP is obtained [N. M. Pattnaik, et al., B. B. A. 530, 428-438, (1978) et al]. Further, the so-called purified CETP is prepared by further purifying the crude CETP with an affinity chromatography [H. Kato, et al., J. Biol. Chem. 264/7, 4082-4087 (1989)]. Lastly, the objective immunogen is prepared by SDS treatment. More specifically, for example, crude CETP or purified CETP prepared by the following method may be used.

Specifically, ammonium sulfate is added to human fresh

plasma to make a 50% saturation solution and the solution was stirred at 4°C for 1 to 2 hours. After that, said solution is centrifuged at 7000rpm for thirty minuets to give a precipitation. The precipitation is dissolved in 10mM phosphate buffer solution (0.15M NaCl, 2mM EDTA, ph: 7.4) and the solution is dialyzed against the same buffer at 4°C overnight. Then, the specific gravity of the solution is adjusted to 1.21 to 1.25g/ml by dissolving NaBr in the solution. The solution is then ultracentrifuged with ultra centrifugal at 255000 Gav, at 16°C, for 17hours and the lower layer fraction of the specific gravity of 1.21g/ml or more is recovered. The recovered fraction is applied to a phenyl cephalose CL-4B column (φ: 2.6X60cm, Pharmacia Ltd.), which was equilibrated in advance with 10mM Tris-HCl buffer solution (2N NaCl, 2mM EDTA, pH: 8.0), whereby CETP is absorbed on the column. After said column is washed with 10mM Tris-HCl buffer solution (2M NaCl and 2mM EDTA, ph: 8.0) of approximate 2 X bed volume, a CETP active fraction is eluted out from the column with 2mM EDTA solution of 2 X bed volume. The obtained CETP active fraction is added with 0.5mM acetate buffer solution (ph4.5) of 1/9 volumes, the mixture is applied to a CM-cellulose column (CM-52)(φ2.5X17cm, Wattman, Inc.). After the column absorbing CETP is washed with 50mM acetate buffer solution(pH4.5) of approximate 10 X bed volume. A crude CETP can be obtained after the active fraction is eluted out with 50mM acetate buffer solution (90mM NaCl, pH4.5) of approximate 10 X bed volume. Further, after thus obtained crude CETP is dialyzed against 39mM phosphate buffer solution (0.025% EDTA, pH6.8), CETP is absorbed on a cephalose 4B column (φ1.5X18cm,

Pharmacia Ltd.), in which succinylated low specific gravity lipoprotein (LDL) was immobilized and the column was equilibrated with the phosphate buffer solution. Next, the column containing CETP is washed with said buffer solution of 5 X bed volume, then the purified CETP can be obtained by eluting out the active fraction from the column with 4mM phosphate buffer solution (pH6.8) of 2 X bed volume.

For the method of SDS treatment for CETP obtained by the above-mentioned method, there are following example methods: CETP and SDS are mixed and the mixture is heated to make CETP negatively charged, at usually 15 to 100°C, preferably 25 to 100°C, more preferably 37 to 100°C, for usually 2 minutes to 20 hours, preferably 2 minutes to 2 hours, more preferably 2 minutes to one hour. Another example is that CETP is electrophoresed on a SDS polyacrylamide gel according to a well-known method to make CETP negatively charged, CETP fraction is cut out from said poly-acryl amide gel and is homogenized ["Useful immune examination" 9 to 10, edited by K. Shimada, published by Kodansha]. In further embodiment, the above-mentioned two methods may be combined.

Using SDS treated CETP obtained with the above-mentioned methods, the anti-CETP antibody of the present invention may be obtained as follows.

Firstly, a suspension is produced by mixing the SDS treated CETP obtained with the above-mentioned methods with adjuvant such as complete Freund's adjuvant. Adequate volume of the suspension is injected into the above-mentioned adequate animals. For example, a dosage amount equal to the CETP amount of usually 0.1 to 100μg, preferably 0.1 to 10μg,

is injected to the animals with an interval of usually every 1 to 5 weeks, preferably every 2 to 5 weeks and the injections are repeated usually 3 to 10 times, preferably 3 to 8 times, via subcutaneous, vein, or abdominal cavity of the animals to be immunized. After the immunization, the blood is collected from said animals and the reaction between the antisera and the SDS treated CETP is confirmed by a well-known method such as the solid phase enzyme immunoassay method (ELISA method), in which SDS treated CETP is used as the solid phase. After confirmation, the spleen is removed from the immunized animal after 3 days from the last immunity, and the spleen cell is prepared with conventional method. The obtained spleen cell and myeloma cell such as NS-1 cell are fused with conventional method, and the cell fusion is HAT selected with the conventional method. The selected fused cell is cultured, and the supernatant of the culture is subjected to an ELISA assay in which the SDS treated CETP is used as the solid phase, or the supernatant is applied to an immunostaining procedure using western-blot protocol on a polyvyniliden difluoride (PVDF) membrane after SDS -polyacrylamide gel electrophoresis, to further select the cell which produces an anti-CETP antibody showing the above-mentioned properties. Further, cloning is performed twice after limiting dilution to select the anti-CETP monoclonal antibody producing hybridoma strain which is judged to produce high titer antibody. Then, by ordinarily procedure, the obtained hybridoma is injected into animal's abdominal cavity to produce the anti-CETP in antibody in the abdominal dropsy. The abdominal dropsy is collected and purified with purifying method ordinarily used in this

field such as ammonium sulfate salting-out, dialysis against buffer such as phosphate buffer solution, chromatograph on DEAE cellulose column, protein A column or the like, to obtain purified monoclonal antibody. In addition, the judgment for subclass of monoclonal may be performed with well-known methods such as double immuno-diffusion (Kimbara Publishing, Ltd. 30th ed. 842-843). For more specifically, as preferable anti-CETP antibodies of the present invention, CM5a-27 produced by hybridoma CM5a-27 and CM5a-39 produced by hybridoma CM5a-39 are exemplified. Hybridoma CM5a-27 and hybridoma CM5a-39 are deposited to National Institute of Bioscience and Human-Technology, Agency Industrial Science and Technology, Department of Trade and Industry as Deposition No. FERM P-15156 and FERM P-15157 respectively.

By using the anti-CETP monoclonal antibody of the present invention, CETP in a sample can be measured more simply and with higher precision than the conventional method with immunoassay method.

The immunoassay method of CETP of the present invention may be adapted to any well-known immunoassay protocols such as enzyme immunoassay (EIA), radio immunoassay (RIA), fluorescence immunoassay (FIA), immuno-nephelometry, and immunomephelometry, excepting for forming an antigen-antibody complex between the anti-CETP antibody and the CETP in the SDS treated sample under the presence of SDS. As to the other reagents, those well known in the art may be used and selected as appropriate.

The sample analyzed by the immunoassay of CETP according to the present invention is not particularly limited insofar

as the sample includes CETP, e.g. fluid such as blood plasma, serum or cell tissue liquid.

To perform the method of the present invention, firstly, the sample is treated with SDS. The method for treating sample with SDS is not particularly limited if the method can make CETP in the sample negatively charged by using SDS. For example, SDS solution is mixed with a sample and the mixture is heated. When the mixed is heated, for example, SDS is mixed with the sample, then the obtained mixture is heated, ordinarily at 15 to 100°C, preferably 25 to 100°C, more preferably 37 to 100°C, and ordinarily for 2 minutes to 20 hours, preferably 2 minutes to 2 hours, more preferably 2 minutes to 1 hour. The concentration of SDS is not particularly limited if CETP in the sample can be made negatively charged. For example, in the method in which SDS solution is mixed with the sample and the mixture is heated, the concentration of SDS in the mixture may be selected from those in the range of 0.001 to 10% (W/V), preferably 0.01 to 1% (W/V).

The SDS solution for treating the sample with SDS may be prepared based on any solvents, provide that the solvent does not prevent that CETP in the sample from being made negatively charged. For example, (1) Purified water, (2) phosphate buffer solution, Tris-HCl buffer solution, BES buffer solution, Good buffer solution such as MOPS buffer solution and the like with pH5 to 9, preferably pH6 to 8, 1 to 500mM, preferably 1 to 50mM, are preferable as the solvent. Further, salts such as NaCl, a surface-active agent, and a preservative may be included in the solution if they are not contained in a quantity which prevents that CETP in the sample

from being made negatively charged.

The anti-CETP antibody used in the immunoassay method of the present invention may be any antibody if the antibody is reacted specifically with SDS treated CETP, that is, is not reacted, or has low reactivity to untreated CETP. In other words, the antibody may be the one that scarcely reacts with untreated CETP or has higher reactivity for SDS treated CETP than for untreated CETP. Preferably the antibody should have the above property and show no cross-reactivity or only show a low level of cross-reactivity to other proteins or a measurement preventing material in blood such as albumin, immunoglobulin, and fibrinogen , i.e. showing no substantial cross-reactivity hindering the measurement in practical use. Further, the anti-CETP antibody that can be used in immunoassay method of the present invention may be a polyclonal antibody or a monoclonal antibody derived from animals such as horse, cow, sheep, goat, rabbit, guinea pig, rat, and mouse, insofar as the antibody has the above-mentioned properties. However, monoclonal antibodies are preferable in consideration of specificity and stable quality etc. For such monoclonal antibodies, anti-CETP monoclonal antibody which is produced by the hybridoma obtained by the above-mentioned process may preferably be used. Above all, the monoclonal antibody CM5a-27 produced by hybridoma CM5a-27 and the monoclonal antibody CM5a-39 produced by hybridoma CM5a-39 are preferable. Further, these anti-CETP antibodies may be used alone or in combination.

In the immunoassay method of CETP of the present invention, the reaction to form an antigen-antibody complex

between the CETP and the anti-CETP antibody is performed under the presence of SDS. Further, the amount of SDS is not particularly limited to a certain value if it does not hinder the formation of the antigen-antibody complex. However, if the concentration of SDS is too low, the antigen-antibody complex would not be sufficiently formed, while if the concentration is too high, it may prevent the antigen and antibody from forming the complex. Therefore, the concentration of SDS for reacting CETP with the anti-CETP antibody may be selected from those in the range of from usually 0.001 to 0.3% (W/V), preferably from 0.01 to 0.1% (W/V), more preferably from 0.02 to 0.03% (W/V). Further, with regard to the reaction conditions for forming the antigen-antibody complex between CETP and the anti-CETP antibody under the presence of SDS, there is no particular limitation and the protocol may be designed based on any conventionally used reaction conditions. The reaction conditions may usually be at 4 to 40°C, preferably 25 to 37°C, and usually for 0.5 to 48 hours, preferably 1 to 5 hours. In addition, if SDS concentration in the reaction solution is too high, for example 0.1% and more, in such case the reaction should be performed at above 15°C because if the reaction is performed at below 15°C, SDS may separate out.

An example of the immunoassay method for CETP of the present invention is as described below. Specifically, a SDS treated sample as prepared in the above-mentioned process is reacted with a certain amount of anti-CETP antibody (Primary antibody) in the presence of SDS. Specifically, after forming the antigen-antibody complex (CETP in the sample : anti-CETP

antibody complex) in an adequate solution at 4 to 40°C for 0.5 to 48 hours under the presence of SDS, the resultant reaction solution is contacted with an insoluble carrier containing CETP immobilized thereon. Then, the further reaction is carried out at 4 to 40°C for 0.5 to 48 hours under the presence of SDS so that the free anti-CETP antibody in the reaction solution is reacted with the immobilized CETP to form an antigen-antibody complex (immobilized CETP : anti-CETP antibody complex) on the insoluble carrier. Further the insoluble carrier is washed in accordance with conventional method and the washed carrier is contacted with a labeled antibody against the anti-CETP antibody (secondary antibody; a labeled anti-mouse IgG antibody in the case that the afore-mentioned anti-CETP antibody is a mouse monoclonal antibody, for example) at 4 to 40°C for 0.5 to 16 hours to form a labeled antibody-antigen complex (immobilized CETP : anti-CETP antibody : labeled antibody complex). The amount of the label on the carrier is then analyzed. The concentration of CETP in the sample can be determined based on the amount of the label with using a standard curve showing the correlation between the amount of the label and the concentration of CETP that has been generated by using a solution containing a known-amount of CETP and using the same reagents and conditions. The above-mentioned example is a measuring method based on competition protocol. However, by the measuring methods based on noncompetitive protocol, so-called sandwich method, the measuring method can be performed undoubtedly.

In the CETP measuring method of the present invention,

the deactivated CETP can be used as the standard because the anti-CETP antibody of the present invention does not recognize the lipid transfer catalytic site in the CETP molecule. Therefore, in the CETP measuring method of the present invention, the CETP measurement is more precise than that of the conventional method which cannot help using CETP standard showing lipid transfer activity (i.e. extremely unstable).

The insoluble carrier that may be used in the above-mentioned measuring method may include any carriers that may also be used in the conventional immunoassay method. The carrier may preferably be a synthetic high polymer such as polystyrene, polypropylene, polyvinyl chloride, polyethylene, polychloro carbonate, silicone resin, and silicone rubber, and inorganic substance such as porous glass, frosted glass, alumina, silica gel, activated carbon, and metal oxide. Further, these insoluble carriers can be used in various forms such as tube, beads, disk-shaped piece, micro particle (latex particle), and microplate. Above all, a microplate is more preferably used from the point of view of the easiness of washing and handling of many samples at the same time. In addition, when the microplate is used as an insoluble carrier, the measurement can be more simply carried out by using a microplate reader to measure the amount of the label. Further, for immobilizing the antigen to the insoluble carrier, a well known immobilizing method such as immobilizing method by covalent binding, or immobilizing method by physically attaching (Patent Publishing No.H5-41946) may well be used.

In the measuring method of the present invention, the label, which can be used for labeling antibody against

anti-CETP antibody (secondary antibody) used for detecting the amount of antigen-antibody complex between CETP and the anti-CETP antibody or for producing the labeled anti-CETP antibody used for detecting CETP by sandwich method, includes an enzyme such as peroxidase, microperoxidase, acid phosphatase, alkaline phosphatase, beta-galactosidase, glucose oxidase, glucose-6-phosphate dehydrogenase, acetylcholine esterase, malic acid dehydrogenase, luciferase; a radio isotope such as ^{99m}Tc, ^{131}I, ^{125}I, ^{14}C, ^{3}H; a fluorescent material such as fluorescein, dancyl, fluorescamine, coumarin, naphtylamine or the derivatives thereof; a luminescent material such as luciferin, isoluminol, luminol, bis-(2,4,6-triphrolophenyl) oxalate; a material having an absorption in the ultraviolet range such as phenol, naphthol, anthracene, or the derivatives thereof; a material with properties as spin label reagent, e.g. a compound having oxyl group such as 4-amino-2,2,6,6-tetrametyl piperidine-1-oxyl, 3-amino-2,2,5,5-tetrametyl pyrolidine-1-oxyl,2,6-di-t-butyl-α-(3, 5-di-t-butyl-4-oxo-2, 5-cyclohexadiene -1-ylidene) -p-tolyloxyl. Such all labeling materials conventionally used in this field may also be used.

Further, for labeling the antibody against the anti-CETP antibody or the anti CETP antibody, any conventional labeling method employed in a well-known EIA , RIA or FIA protocol [" Medical chemistry experimental course" 8, Y. Yamamura, supervising editor, 1st ed. Nakayama Shoten, Ltd. 1971; "Diagram explained fluorescent antibody" A. Kawao, 1st ed. Softscience Co. Ltd. 1983; "enzyme immunoassay" E.

Ishikawa, T. Kawai and K. Muroi edited, 2nd ed. Igaku Shoin, Ltd 1982 etc.] may be used. Further, as labeling method, the conventional method which uses reaction between avidin (or streptoavidin) and biotin may well be used.

The solution used for forming the antigen-antibody complex in the method of the present invention may be any solution if the solution does not prevent the formation of the complex. Buffers as conventionally used in this field, such as phosphate buffer solution, Tris-buffer solution, BES buffer solution, and Good's buffers such as MOPS buffer solution with pH 5 to 9, preferably pH 6 to 8, and 1 to 500mM, preferably 1 to 50mM, may be used as the solution. In addition, to use the solution for forming the antigen-antibody complex between the anti-CETP antibody of the present invention and CETP, it goes without saying that SDS must be included in the range of concentration as above-mentioned in said solution. In addition, in the solution, materials ordinarily used in this field such as carbohydrates, proteins, surface-active agents may be included in the range of concentration ordinarily used in this field.

Further, the determination of the amount of the label presented on the labeled antigen-antibody complex on behalf of the labeled antibody against anti-CETP antibody (secondary antibody) or the labeled anti-CETP antibody may depend on the detectable property of the used label and an appropriate method known to the art may be used for that purpose. For example, when the label is an enzyme, the measurement may be performed according to the conventional EIA method and according to the method described in ["Enzyme immunoassay" Protein, nucleic

acid and enzyme, addition volume No.31, T. Kitagawa, T. Nambara, A. Tsuji, and E. Ishikawa ed. 51-63, Kyoritu Shuppan, Co. Ltd. 1987]. When the label is a radioactive material, the measurement may be performed according to the conventional RIA method by considering the kind and intensity of radiation from said radioactive material and an appropriate measuring instrument such as immersion GM counter, liquid scintillation counter, well-type scintillation counter, and HPLC counter [" Medical chemistry experimental course" 8, Y. Yamamura, supervising editor, 1st ed. Nakayama Shoten, 1971 etc.] may be selected and used. Further, when the label is a fluorescent material, the measurement may be performed according to the conventional FIA method using measuring instrument such as a fluorometer and according to the method described in ["Diagram explained fluorescent antibody" A. Kawao, 1st ed. Softscience Co. Ltd. 1983]. When the label is a luminescent material, the measurement may be performed according to the conventional method using a measuring instrument such as photocounter and according to the method described in ["Enzyme immunoassay" Protein, nucleic acid and enzyme, addition volume No.31, T. Kitagawa, T. Nambara, A. Tsuji, and E. Ishikawa ed. 252-263, Kyoritu Shuppan, Co. Ltd. 1987]. Further, when the label is a material with absorption in the ultraviolet range, the measurement may be performed by the ordinarily method using a measuring instrument such as spectrophotometer, and when the label has the property of spin, the measurement may be performed according to any conventional methods using an electro-spin resonance instrument in accordance with a method as described in ["Enzyme immunoassay" Protein, nucleic acid and enzyme,

addition volume No.31, T. Kitagawa, T. Nambara, A. Tsuji, and E. Ishikawa ed. 264-271, Kyoritu Shuppan, Co. Ltd. 1987}.

More specifically, when label is an enzyme, a well-known method in which the labeling material is reacted with coloring reagent to lead to luminescent reaction and subsequent generating dye quantity is measured with spectrophotometer, may be used. For coloring reagents used for such purpose, the conventional coloring reagents in this field such as o-phenylene diamine, o-nitrophenyl-β-D-galactoside, 2,2'-adino-bis (3-ethyl benzethiazorine-6-sulfonic acid) (ABTS), N-ethyl-N-sulphopropyl-m-anisidine (ADPS), and p-nitrophenyl phosphate, may be used. Further, for stopping the fluorescent reaction, the reaction stopping step ordinarily used in this field such as a step of adding to the reaction solution, an enzymeinhibitor, for example, 1-6N sulfuric acid, may be included.

The CETP measuring reagent comprising the anti-CETP antibody of the present invention which is used for measuring CETP in a sample, may be prepared by incorporating other components as used in the conventional immunoassay and the concentrations of the other components may be those as usually employed in this field, provided that the anti-CETP antibody with properties as above-mentioned is included. The preferable aspect and the concentration and the like of other components are as mentioned in the above.

The CETP measuring reagent comprising the anti-CETP antibody of the present invention may be used in various immunoassay methods as above-mentioned.

When immunoassay method for CETP of the present invention

is performed, a CETP measuring kit comprising necessary reagents in combination may be used. Examples of the kit include those comprising SDS and an anti-CETP antibody in combination. More specifically, for example, when the kits are for use in an immunoassay using CETP immobilizing plate, the kit may include (i) a CETP immobilizing plate, (ii) a CETP solution for generating a standard curve, (iii) a sample treating solution containing SDS, (iv) a reagent consisting of an antibody against the anti-CETP antibody, (v) a reagent consisting of an enzyme-labeled antibody against the anti-CETP antibody (hereinafter referred to as " reagent containing labeled antibody"), (vi) a coloring reagent for detecting labeled enzyme, (vii) an enzyme-reaction stopping reagent. The solutions or reagents of the kit may include any components such as carbohydrates, proteins and stabilizing materials and the like such as surface-active agents etc., in an amount as conventionally used in this field insofar as they do not adversely affect the reaction. Further, the reagent containing the anti-CETP antibody and the reagent containing the labeled antibody, etc. may be in a form of solution or freeze-dried solid. In the kit, the freeze-dried solids are preferably accompanied by a solution for dissolving the freeze-dried solids. The reagents containing the anti-CETP antibody and the reagents containing the labeled antibody and the solutions for dissolving freeze-dried solids may further comprises a buffer, a preservation agent, a stabilizing agent etc., within the concentration used ordinarily in the field.

The preferred references and embodiments of the present invention will be more specifically described. It should be

noted, however, that the present invention is not limited to those embodiments.

[Example]

[Reference Example 1] Purification of CETP

Ammonium sulfate was added to 1000ml of human fresh blood plasma sample to bring the sample to 50% saturation and the sample was stirred at 4°C for one hour. The sample solution was centrifuged at 7000rpm/min at 4°C for 30minutes. The resultant precipitate was dissolved with approximately 100ml of 10mM phosphate buffer solution (0.15M NaCl, 2mM EDTA, pH7.4)(hereinafter referred to as "PBS buffer") and dialyzed at 4°C against PBS buffer over night (approximately 5l x 2 times). Then, the dialyzed solution was adjusted to the specific gravity of 1.21 to 1.25g/ml with an appropriate amount of NaBr and centrifuged at 255000 x Gav at 16°C for 17 hours, and the lower layer fraction (fraction of the specific gravity of 1.21g/ml and more) was collected. The collected lower layer fraction was applied to a phenylcephalose CL-4B column (φ2.6 x 60cm, Pharmacia) pre-equilibrated with 10ml Tris-HCl buffer solution (2M NaCl, 2mM EDTA, pH8.0) so that CETP is absorbed on the column. Then, the column was washed with approximately 700ml of said buffer, and the CETP fraction was eluted with 2mM EDTA aqueous solution. After a 1/9 volume of 0.5M acetate buffer solution (pH4.5) was added and mixed with the eluted CETP fraction, the mixture was applied to a CM-cellulose (CM-52) column (φ2.5 x 17cm, Wattman) pre-equilibrated with 50mM acetate buffer solution (pH4.5) so that CETP is absorbed on the column. After said column was washed approximately 500ml of 50mM acetate buffer solution, CETP was eluted with

approximately 500ml of said buffer solution further including 90mM NaCl to obtain crude CETP fraction. Then, the obtained crude purified CETP fraction was dialyzed against a 39mM phosphate buffer solution (0.025% EDTA, pH6.8), followed by the chromatography on a cephalose 4B column (φ1.5 x 18cm, Pharmacia) pre-equilibrated with a 39mM phosphate buffer solution (0.025% EDTA, pH6.8) and containing succinylated low specific gravity lipoprotein (LDL) immobilized thereon, whereby CETP is absorbed on the column. After said column was washed with approximately 150ml of 39mM phosphate buffer solution (0.025% EDTA, pH6.8), CETP fraction was eluted with 4mM phosphate buffer solution (pH6.8) and CETP is collected. Incidentally, the crude CETP fraction as obtained in the above, purified CETP fraction (eluted fraction from the succinylated LDL column with 4mM phosphate buffer solution), a control fraction which was not absorbed on the succinylated LDL immobilized cephalose column (succinylated LDL column non-absorbed fraction) and a control fraction consisting of effluent with 1mM phosphate buffer (pH6.8) (succinylated LDL column 1mM phosphate buffer solution eluted fraction) were subjected to an electrophoresis on 10% SDS-polyacrylamide gel (SDS -PAGE) [New ed. "Electrophoresis experiment" H. Hirai et al., supervised editor, Electrophoresis Academy ed. 288-298, 1998, Bunkosha, Co. Ltd]. The results to demonstrate the existence of CETP is shown in Fig. 1. [Silver stain II kit WAKO(made of Wako Pure Chemical Industries, Inc) was used for silver stain method to detect each proteins].

Each lane numbers in Fig. 1 shows the results of using following samples respectively.

Lane A: molecular weight marker (SDS-PAGE Standard "low": Biorad).

B: Crude CETP

C: Succinylated LDL column non-absorbed fraction.

D: Purified CETP (eluted fraction form the succinylated LDL column with 4mM phosphate buffer solution).

E: Succinylated LDL column 1mM phosphate buffer solution eluted fraction.

Further, in Fig. 1, arrow shows CETP region.

[Example 1] Preparation of antibody anti-monoclonal

After the CETP fraction obtained in the Reference Example 1 and molecular weight marker (SDS-PAGE Standard "low": Biorad) were subjected to 10% SDS-PAGE, only polyacrylamide gel applied the molecular weight marker thereon was CBB stained with CBB staining reagent (Wako Pure Chemical, Inc). Then, polyacrylamide gel on which CETP fraction was applied, was washed twice with approximately 100 ml of distilled water, and the portion corresponding to CETP fraction of approximately 74,000 molecular weight based on the molecular weight marker stained by CBB was cut out form the gel in approximately 1mm in width. The cut-out gel (for 10 lanes) was added with 2ml of PBS buffer solution and the mixture was pushed out from the syringe with 22G needle several times to crush gel to obtain an antigen solution. Then, the antigen solution was processed in accordance with the manufacture's instructions of Ribi adjuvant system MPL+TDM emulsion (Ribi Immuno Chem Research, Inc) to a suspension containing the immunogen. Immunization was carried out with this immunogen by injecting it into abdominal cavity of mouse so as to be 0.1 to 10μg/mice of CETP,

according to a standard operation method. Second immunization was performed after three weeks from first immunization, and last immunization was performed after two weeks from second immunization. The spleen was removed from the mouse after three days from last immunization, and the spleen cells were obtained by grinding the tissue on a sterilized frosted glass. The spleen cells were suspended in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd.) and centrifuged. These operations were repeated several times, each followed by thoroughly washing. 1.5×10^8 cells of the washed spleen cells above and 1.5×10^7 cells of mouse myeloma (P3-NS-1-Ag4(NS-1) pre-washed also with RPMI 1640 medium, were combined in a test tube and mixed. After the mixing, the cells were spread on the bottom surface of the test tube. Then, 1ml of polyethylene glycol 6000 (Wako Pure Chemical Industries, Inc.) solution adjusted to 50%(W/V) with RPMI 1640 medium, was gently added into the test tube. The cell fusion reaction was carried out for one minute. Then, 11ml of RPMI 1640 medium was gradually added to the test tube to dilute PEG, thereby the fusion reaction was terminated. Thus obtained cell suspension was centrifuged at 1,500rpm for five minutes and the supernatant liquid was removed. Then, the residual cells were suspended in 100ml of RPMI 1640 medium supplemented with 10% fetal calf serum. A 0.1 ml of the suspension was dispensed to each well of a 96 well-microplate and incubated for 24 hours at 37°C under the atmosphere of 5%CO_2. After the incubation for 24 hours, a 0.1ml of 2 X (as compared to the standard concentration) HAT medium was dispensed to each well. After further incubation for approximately 48 hours, the culture medium in each well was

replaced by standard HAT medium. After one week from the cell fusion, a 0.1ml of the culture supernatant was removed from each well and a 0.1ml of fresh HT medium is added thereto. This step was repeated on the next day. After 10 days from the cell fusion, the culture supernatant was tested for the antibody titer by using ELISA method with purified CETP as the solid phase, thereby cells in a well with high antibody titer were cloned by suing the limiting dilution method. Further, thus obtained medium from the cloning culture was tested for the specificity by using an immunostaining procedure [Towbin., et al., Proc. Natl. Acad. Sci. USA., 76, 4350 (1979)] based on western blotting method. Three types of clones (CM5a-27, CM5a-31 and CM5a-39) which produce antibodies with high titer and specificity were obtained.

[Example 2] Study on cross-reactivity to measurement preventing materials

The cross activity of each monoclonal antibodies produced by the 3 clones in Example 1 to other proteins (measurement preventing materials) in blood was studied with immunostaining procedure [Towbin., et al., Proc. Natl. Acad. Sci. USA., 76, 4350 (1979)] by using human IgG, human albumin and human fibrinogen as the other proteins in blood respectively. Further, TP-3 [Swenon, T., et al., J. Bio. Chem. 264, 14318-14326 (1989)] as a conventional monoclonal antibody, and a commercially available monoclonal anti-CETP antibody (Shibayaki, Inc) were also involved as the controls for testing the cross-reactivity to other proteins in blood. The results are shown in Table 1. In Table.1, double-circles, circle, triangle, and X show reactivity to each protein in blood wherein

Double-circles indicate very strong reactivity, circle indicates strong reactivity, triangle indicates week reactivity and X indicates no reactivity.

Further, the antibody titers of these five anti-CETP monoclonal antibodies were evaluated by using an ELISA method with purified CETP as the solid phase. These results are also shown in Table 1. The titers in Table1 show relative value to the titer of TP-3 as 1.

[Table 1]

Item	TP-3	Commercial Product	CM5a-27	CM5a-31	CM5a-39
Human IgG	◎	△	△	△	×
Human albumin	○	×	×	×	×
Human phibrinogen	○	×	×	×	×
Titer	1	1/4	30	1/2	15

As is clear from the result of Table 1, as compared to TP-3, it was found that the anti-CETP monoclonal antibody of the present invention showed weak cross-reactivity to other proteins, i.e.measurement preventing materials in blood. Further, it was found that the commercially available anti-CETP monoclonal antibody had weak cross-reactivity to other proteins in blood, its titer to CETP was, however, extremely low. On the other hand, as compared to TP-3 and the commercially available monoclonal antibody, it was found that CM5a-27 and CM5a-39 among the anti-CETP monoclonal antibody of the present invention showed extremely high titer to CETP. Based on foregoing, it can be concluded that the monoclonal antibody of the present invention is most suitable for theCETP

immunoassay because its cross-reactivity to measurement preventing materials was extremely low and it showed higher titer to CETP. Incidentally, among the above-mentioned clones, CM5a-27 and CM5a-39 were deposited to National Institute of Bioscience and Human-Technology, Agency Industrial Science and Technology, Department of Trade and Industry and the Deposition Number and Deposition Date are as described below.

Clone CM5a-27: Deposition Number; FERM P-15156 Deposition Date: 6 Sept. 1999

Clone CM5a-39: Deposition Number; FERM P-15157 Deposition Date: 6 Sept. 1999

[Example 3] CETP measurement in samples

(1) Preparation of CETP immobilizing plate

To each well of a 96 well-microplate, 100μl of 50mM carbonic acid buffer solution(pH9.6) containing 0.2μg/ml of the purified CETP in the Reference Example 1 was dispensed and the purified CETP was immobilized in the well at 4°C for 16 hours. Then, each well was blocked with 200 μl of PBS buffer solution containing 1% BSA at 37°C for one hour and washed four times with PBS buffer solution to obtain the CETP immobilized plate.

(2) Pretreatment of samples

Twenty-three plasma samples from the patients with protopathic biliary cirrhosis (PBC) and twenty plasma samples from healthy subjects were included. A 10μl of 0.5% SDS aqueous solution was added to a 10μl portion of each sample and reacted at 37°C for one hour. The obtained reaction solution was mixed with 200μl of 10mM phosphate buffer solution(PB buffer

solution) (1% BSA, pH7.4) containing 17ngAb/ml of CM5a-27 monoclonal antibody (primary antibody), and reacted at 37°C for one hour to prepare the treated sample.

(3) Measurement of CETP in samples

A 50μl of the treated sample in (2) and a 50μl of 0.023% SDS aqueous solution were mixed in the well of CETP immobilized plate as prepared in (1), and the mixture was reacted at 37°C for two hours (reaction between unreacted CM5a-27 monoclonal antibody and CETP on the plate). After said plate was washed with PBS buffer four times, a 100μl of PB buffer solution containing 1.3μg Ab/ml of horseradish peroxidase labeled rabbit anti mouse immuno-globulin polyclonal antibody (secondary antibody, Dako Japan Inc.) was dispensed to the well and reacted at 37°C for one hour. After reaction, said plate was washed with PBS buffer solution four times. Then, a 100μl of coloring liquid (containing 0.017% H_2O_2 and 50mM citric acid in 100mM phosphate buffer solution, pH4.8) containing 3mg/ml of o-phenylenediamine was dispensed to the well, and the enzymatic reaction was performed at room temperature for 30 minutes. Then, a 100μl of 6N sulfonic acid was added to the well to stop the reaction. Absorbance of each well was measured with microplate reader UVmax (Molecular Devices corporation), condition-set to λ=490nm, end-point measuring, using SOFTmax-j (Ver.2.2, Wako Pure Chemical Industries). The CETP concentration in the sample was calculated from the measured absorbance based on a standard curve indicating the correlation between CETP concentration in the sample and absorbance from the label. The standard curve was prepared by adding purified CETP to the PB buffer solution to prepare

the solutions containing 0, 1.5, 3, 6, 9, 12μg/ml of CETP, respectively. These solutions with each concentration were mixed with the sample. Except this preparation, the process was performed using same operation and reagents as mentioned in the above.

(4) Results

Fig. 2 shows the standard curve prepared by using standard CETP solution. Table.2 shows the concentration of CETP in each sample calculated based on the standard curve. Further, Fig. 3 shows the significant difference between PBC patients and healthy subjects in terms of the concentration of CETP.

Sample No.	PBC patient	Normal subject
1	4.1	3.6
2	4.3	3.6
3	3.9	3.7
4	5.7	2.6
5	4.3	3.7
6	4.6	3.3
7	3.9	3.1
8	2.4	2.8
9	4.4	3.9
10	3.3	3.6
11	4.4	3.1
12	3.8	3.3
13	4.1	3.8
14	5.0	3.3
15	3.3	3.5
16	3.2	3.3
17	4.5	3.6
18	4.1	3.3
19	5.0	3.9
20	3.7	3.2
21	4.6	—
22	4.1	—
23	4.3	—
n	23	20
MEAN	4.13	3.41

[Table 2]

As seen from the results of Table 2 and Fig. 3, the difference in the CEPT concentration between the PBC patients and the healthy subjects is significant. Therefore, it can be concluded that the CETP concentration in each patient's blood plasma determined by using the measuring method of the present invention is useful in PBC diagnosis.

[Effect of the invention]

As above-mentioned, the present invention provides an anti-CETP monoclonal antibody, which has high titer to CETP and is a low level of cross-reaction to other proteins (measurement preventing materials) in blood of, an antibody reacting specifically with SDS treated CETP, an immunoassay method for CETP in sample using said antibody, and CETP measuring reagents for use in the method. The present invention much contributes to industry because the invention accomplished the effect to measure the antigen more simply and with higher precision as compared to conventional methods, by using CETP measuring method by use of the anti-CETP antibody of the present invention.

[The Brief Description of the Drawings]

Fig. 1 shows the results in which each fraction obtained in the Reference Example 1 was subject to 10% SDS-polyacrylamide gel electrophoresis.

Fig. 2 shows a standard curve indicating the correlation between the concentration of cholesteryl ester transfer protein (CETP) obtained in Example 3 and absorbance (OD.490nm).

Fig. 3 shows the results of significant difference test between protopathic biliary cirrhosis (PBC) patient plasma in Example 3 and normal plasma.

[Description of Symbols]

In Fig. 1, each lane number shows the result in which the following samples were subject to 10% SDS-polyacrylamide gel electrophoresis.

Lane A: molecular weight marker (SDS-PAGE Standard "low": Biorad).

B: Crude CETP

C: Succinylated LDL column non-absorbed fraction.

D: Purified CETP (eluted fraction form the succinylated LDL column with 4mM phosphate buffer solution).

E: Succinylated LDL column 1mM phosphate buffer solution eluted fraction.

Further, in Fig. 1, arrow shows CETP region.